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GENERATION OF PLANTS WITH IMPROVED DROUGHT TOLERANCE

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REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. provisional patent application 60/482,139
5 filed 6/24/2003, the contents of which are hereby incorporated by reference.

BACKGROUND OF THE INVENTION

Crop production is affected by numerous abiotic environmental factors, with soil salinity and drought having the most detrimental effects. Approximately 70% of the 10 genetic yield potential in major crops is lost due to abiotic stresses, and most major agricultural crops are susceptible to drought stress. Attempts to improve yield under stress conditions by plant breeding have been largely unsuccessful, primarily due to the multigenic origin of the adaptive responses (Barkla et al. 1999, *Adv Exp Med Biol* 464:77-89).

15 Considerable effort has focused on the identification of genetic factors that contribute to stress tolerance and on the genetic engineering of crop plants with increased stress tolerance. A number of genes have been identified whose expression or mis-expression is associated with drought tolerance, via a variety of different mechanisms. For instance, transformed tobacco that express maize NADP-malic enzyme display increased 20 water conservation and gained more mass per water consumed than wild-type plants (Laporte et al. 2002, *J Exp Bot* 53:699-705). Significant research effort has focused on the plant hormone abscisic acid (ABA), which is involved in adaptation to various environmental stresses. Transgenic tobacco and transgenic *Arabidopsis* that overexpress 25 the enzyme 9-cis-epoxycarotenoid dioxygenase (NCED), which is key to ABA biosynthesis, display improved drought tolerance (Qin et al. 2002, *Plant Physiol* 128:544-51; Iuchi et al. 2001, *Plant J* 27:325-33). Drought tolerance is often linked to salt tolerance, since both are associated with regulation of osmotic potential and turgor. Accordingly, transgenic plants that overexpress a vacuolar H⁺ pump (H⁺-pyrophosphatase), which generates a proton gradient across the vacuolar membrane, 30 display improved drought- and salt-stress, due to increased solute accumulation and water retention (Gaxiola et al. 2001, *Proc Natl Acad Sci U S A* 98:11444-9). Trehalose also contributes to osmoprotection against environmental stress. Potato plants that mis-express trehalose-6-phosphate synthase, a key enzyme for trehalose biosynthesis, show increased drought tolerance (Yeo et al. 2000, *Mol Cells* 10:263-8).

Arabidopsis has served as a model system for the identification of genes that contribute to drought tolerance. For instance, researchers have identified numerous genes that are induced in response to water deprivation (e.g., Taji et al. 1999, *Plant Cell Physiol* 40:119-23; Ascenzi et al., 1997, *Plant Mol Biol* 34:629-41; Gosti et al. 1995, *Mol Genet* 246:10-18; Koizumi et al. 1993 *Gene* 129:175-82) and *cis*-acting DNA sequences called ABA responsive elements (ABREs) that control ABA or stress responsive gene expression (Giraudat et al. 1994, *Plant Mol. Biol.* 26: 1557).

Several drought tolerant mutants of *Arabidopsis* have been identified. These include the recessive mutants *abh1* (Hugouieux et al. 2001, *Cell* 106: 477), *era1-2* (Pei et al. 1998, *Science* 282: 286) and *abi1-1Ri* (Gosti et al. 1999, *Plant Cell* 11:1897-1909). The mutants *era1-2* and *abh1* were identified by screening for seedlings hypersensitive to ABA, while the mutant *abi1-1Ri* was isolated as an intragenic suppressor of the ABA insensitive mutant *abi1-1*. Dominant drought tolerant mutants were identified by over-expressing *ABF3*, *ABF4* (Kang et al. 2002, *Plant Cell* 14:343-357) or *DREB1A* (Kasuga, 1999 *Nature Biotech* 17: 287). *ABF3* and *ABF4* encode basic-region leucine zipper (bZIP) DNA binding proteins that bind specifically ABREs. *DREB1A* encodes a protein with an EREBP/AP2 DNA binding domain that binds to the dehydration-responsive element (DRE) essential for dehydration responsive gene expression (Liu et al. 1998, *Plant Cell* 10: 1391). A dominant drought tolerant phenotype in tobacco was obtained by over-expressing the soybean *BiP* gene (Alvim et al. 2001, *Plant Physiol* 126, 1042).

Activation tagging in plants refers to a method of generating random mutations by insertion of a heterologous nucleic acid construct comprising regulatory sequences (e.g., an enhancer) into a plant genome. The regulatory sequences can act to enhance transcription of one or more native plant genes; accordingly, activation tagging is a fruitful method for generating gain-of-function, generally dominant mutants (see, e.g., Hayashi H et al., *Science* (1992) 258: 1350-1353; Weigel D, et al., *Plant Physiology* (2000) 122:1003-1013). The inserted construct provides a molecular tag for rapid identification of the native plant whose mis-expression causes the mutant phenotype. Activation tagging may also cause loss-of-function phenotypes. The insertion may result in disruption of a native plant gene, in which case the phenotype is generally recessive.

Activation tagging has been used in various species, including tobacco and *Arabidopsis*, to identify many different kinds of mutant phenotypes and the genes associated with these phenotypes (Wilson K et al., *Plant Cell* (1996) 8: 659-671, Schaffer R, et al., *Cell* (1998) 93: 1219-1229, Fridborg I et al., *Plant Cell* 11: 1019-1032, 1999;

Kardailsky I *et al.*, *Science* (1999) 286: 1962-1965; Christensen S *et al.*, 9th International Conference on *Arabidopsis* Research. Univ. of Wisconsin-Madison, June 24-28, 1998. Abstract 165).

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SUMMARY OF THE INVENTION

The invention provides a transgenic plant comprising a plant transformation vector comprising a nucleotide sequence that encodes or is complementary to a sequence that encodes a DRO3 polypeptide or an ortholog thereof. The transgenic plant is characterized by having increased drought tolerance relative to control plants.

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The invention further provides a method of producing an improved drought tolerance phenotype in a plant. The method comprises introducing into plant progenitor cells a vector comprising a nucleotide sequence that encodes or is complementary to a sequence encoding a DRO3 polypeptide or ortholog thereof and growing a transgenic plant that expresses the nucleotide sequence. In one embodiment, the DRO3 polypeptide has at least 50% sequence identity to the amino acid sequence presented in SEQ ID NO:2 and comprises a TIR domain. In other embodiments, the DRO3 polypeptide has at least 15 80% or 90% sequence identity to or has the amino acid sequence presented in SEQ ID NO:2.

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The invention further provides plants and plant parts obtained by the methods described herein.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Unless otherwise indicated, all technical and scientific terms used herein have the same meaning as they would to one skilled in the art of the present invention.

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Practitioners are particularly directed to Sambrook *et al.* Molecular Cloning: A Laboratory Manual (Second Edition), Cold Spring Harbor Press, Plainview, N.Y., 1989, and Ausubel FM *et al.* Current Protocols in Molecular Biology, John Wiley & Sons, New York, N.Y., 1993, for definitions and terms of the art. It is to be understood that this invention is not limited to the particular methodology, protocols, and reagents described, as these may vary.

As used herein, the term "vector" refers to a nucleic acid construct designed for transfer between different host cells. An "expression vector" refers to a vector that has the ability to incorporate and express heterologous DNA fragments in a foreign cell. Many

prokaryotic and eukaryotic expression vectors are commercially available. Selection of appropriate expression vectors is within the knowledge of those having skill in the art.

A "heterologous" nucleic acid construct or sequence has a portion of the sequence that is not native to the plant cell in which it is expressed. Heterologous, with respect to a control sequence refers to a control sequence (*i.e.* promoter or enhancer) that does not function in nature to regulate the same gene the expression of which it is currently regulating. Generally, heterologous nucleic acid sequences are not endogenous to the cell or part of the genome in which they are present, and have been added to the cell, by infection, transfection, microinjection, electroporation, or the like. A "heterologous" nucleic acid construct may contain a control sequence/DNA coding sequence combination that is the same as, or different from a control sequence/DNA coding sequence combination found in the native plant.

As used herein, the term "gene" means the segment of DNA involved in producing a polypeptide chain, which may or may not include regions preceding and following the coding region, *e.g.* 5' untranslated (5' UTR) or "leader" sequences and 3' UTR or "trailer" sequences, as well as intervening sequences (introns) between individual coding segments (exons) and non-transcribed regulatory sequence.

As used herein, "recombinant" includes reference to a cell or vector, that has been modified by the introduction of a heterologous nucleic acid sequence or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found in identical form within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all as a result of deliberate human intervention.

As used herein, the term "gene expression" refers to the process by which a polypeptide is produced based on the nucleic acid sequence of a gene. The process includes both transcription and translation; accordingly, "expression" may refer to either a polynucleotide or polypeptide sequence, or both. Sometimes, expression of a polynucleotide sequence will not lead to protein translation. "Over-expression" refers to increased expression of a polynucleotide and/or polypeptide sequence relative to its expression in a wild-type (or other reference [*e.g.*, non-transgenic]) plant and may relate to a naturally-occurring or non-naturally occurring sequence. "Ectopic expression" refers to expression at a time, place, and/or increased level that does not naturally occur in the non-altered or wild-type plant. "Under-expression" refers to decreased expression of a polynucleotide and/or polypeptide sequence, generally of an endogenous gene, relative to

its expression in a wild-type plant. The terms "mis-expression" and "altered expression" encompass over-expression, under-expression, and ectopic expression.

The term "introduced" in the context of inserting a nucleic acid sequence into a cell, means "transfection", or "transformation" or "transduction" and includes reference to the incorporation of a nucleic acid sequence into a eukaryotic or prokaryotic cell where the nucleic acid sequence may be incorporated into the genome of the cell (for example, chromosome, plasmid, plastid, or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (for example, transfected mRNA).

As used herein, a "plant cell" refers to any cell derived from a plant, including cells from undifferentiated tissue (*e.g.*, callus) as well as plant seeds, pollen, progaules and embryos.

As used herein, the terms "native" and "wild-type" relative to a given plant trait or phenotype refers to the form in which that trait or phenotype is found in the same variety of plant in nature.

As used herein the term "modified," regarding a plant trait, refers to a change in the phenotype of a transgenic plant relative to the similar non-transgenic plant. An "interesting phenotype (trait)" with reference to a transgenic plant refers to an observable or measurable phenotype demonstrated by a T1 and/or subsequent generation plant, which is not displayed by the corresponding non-transgenic (*i.e.*, a genetically similar plant that has been raised or assayed under similar conditions). An interesting phenotype may represent an improvement in the plant or may provide a means to produce improvements in other plants. An "improvement" is a feature that may enhance the utility of a plant species or variety by providing the plant with a unique and/or novel quality.

An "altered drought tolerance phenotype" refers to detectable change in the ability of a genetically modified plant to withstand low-water conditions compared to the similar, but non-modified plant. In general, improved (increased) drought tolerance phenotypes (*i.e.*, ability to a plant to survive in low- water conditions that would normally be deleterious to a plant) are of interest.

As used herein, a "mutant" polynucleotide sequence or gene differs from the corresponding wild type polynucleotide sequence or gene either in terms of sequence or expression, where the difference contributes to a modified plant phenotype or trait.

Relative to a plant or plant line, the term "mutant" refers to a plant or plant line which has a modified plant phenotype or trait, where the modified phenotype or trait is associated with the modified expression of a wild type polynucleotide sequence or gene.

As used herein, the term "T1" refers to the generation of plants from the seed of T0 plants. The T1 generation is the first set of transformed plants that can be selected by application of a selection agent, e.g., an antibiotic or herbicide, for which the transgenic plant contains the corresponding resistance gene. The term "T2" refers to the generation 5 of plants by self-fertilization of the flowers of T1 plants, previously selected as being transgenic.

As used herein, the term "plant part" includes any plant organ or tissue, including, without limitation, seeds, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores. Plant cells can be obtained 10 from any plant organ or tissue and cultures prepared therefrom. The class of plants which can be used in the methods of the present invention is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledenous and dicotyledenous plants.

As used herein, "transgenic plant" includes reference to a plant that comprises 15 within its genome a heterologous polynucleotide. The heterologous polynucleotide can be either stably integrated into the genome, or can be extra-chromosomal. Preferably, the polynucleotide of the present invention is stably integrated into the genome such that the polynucleotide is passed on to successive generations. A plant cell, tissue, organ, or plant into which the heterologous polynucleotides have been introduced is considered 20 "transformed", "transfected", or "transgenic". Direct and indirect progeny of transformed plants or plant cells that also contain the heterologous polynucleotide are also considered transgenic.

Identification of Plants with an Improved Drought Tolerance Phenotype

We used an *Arabidopsis* activation tagging screen to identify the association 25 between a gene encoding a disease resistance protein (TIR class), which we have designated "DRO3 (for Drought tolerant)," and an improved drought tolerance phenotype. Briefly, and as further described in the Examples, a large number of *Arabidopsis* plants were mutated with the pSKI015 vector, which comprises a T-DNA from the Ti plasmid of 30 *Agrobacterium tumefaciens*, a viral enhancer element, and a selectable marker gene (Weigel *et al, supra*). When the T-DNA inserts into the genome of transformed plants, the enhancer element can cause up-regulation genes in the vicinity, generally within about 10 kilobase (kb) of the insertion. T1 plants were exposed to the selective agent in order to specifically recover transformed plants that expressed the selectable marker and therefore

harbored T-DNA insertions. Seed from transformed plants were planted, grown under adequate water conditions, and then deprived of water. Plants that did not wilt, and that maintained high water content were identified as drought tolerant.

An *Arabidopsis* line that showed increased drought tolerance was identified. The 5 association of the DRO3 gene with the drought tolerance phenotype was discovered by analysis of the genomic DNA sequence flanking the T-DNA insertion in the identified line. Accordingly, DRO3 genes and/or polypeptides may be employed in the development of genetically modified plants having a modified drought tolerance phenotype ("a DRO3 phenotype"). DRO3 genes may be used in the generation of crops and/or other plant 10 species that have improved ability to survive in low-water conditions. The DRO3 phenotype may further enhance the overall health of the plant.

DRO3 Nucleic Acids and Polypeptides

Arabidopsis DRO3 nucleic acid (cDNA) sequence is provided in SEQ ID NO:1 15 and in Genbank entry GI 18404238. The corresponding protein sequence is provided in SEQ ID NO:2 and in GI 15219124. The TAIR designation is Atlg52900.

As used herein, the term "DRO3 polypeptide" refers to a full-length DRO3 protein or a fragment, derivative (variant), or ortholog thereof that is "functionally active," meaning that the protein fragment, derivative, or ortholog exhibits one or more of the 20 functional activities associated with the polypeptide of SEQ ID NO:2. In one preferred embodiment, a functionally active DRO3 polypeptide causes an altered drought tolerance phenotype when mis-expressed in a plant. In a further preferred embodiment, mis-expression of the functionally active DRO3 polypeptide causes improved drought tolerance. In another embodiment, a functionally active DRO3 polypeptide is capable of 25 rescuing defective (including deficient) endogenous DRO3 activity when expressed in a plant or in plant cells; the rescuing polypeptide may be from the same or from a different species as that with defective activity. In another embodiment, a functionally active fragment of a full length DRO3 polypeptide (i.e., a native polypeptide having the sequence of SEQ ID NO:2 or a naturally occurring ortholog thereof) retains one or more of the biological properties associated with the full-length DRO3 polypeptide, such as signaling 30 activity, binding activity, catalytic activity, or cellular or extra-cellular localizing activity. A DRO3 fragment preferably comprises a DRO3 domain, such as a C- or N-terminal or catalytic domain, among others, and preferably comprises at least 10, preferably at least 20, more preferably at least 25, and most preferably at least 50 contiguous amino acids of

a DRO3 protein. Functional domains can be identified using the PFAM program (Bateman A et al., Nucleic Acids Res (1999) 27:260-262; website at pfam.wustl.edu). A preferred DRO3 fragment comprises a TIR domain (PF01582). PFAM analysis identified a TIR domain at amino acids 40 to 159 of SEQ ID NO:2. Functionally active variants of 5 full-length DRO3 polypeptides or fragments thereof include polypeptides with amino acid insertions, deletions, or substitutions that retain one or more of the biological properties associated with the full-length DRO3 polypeptide. In some cases, variants are generated that change the post-translational processing of a DRO3 polypeptide. For instance, variants may have altered protein transport or protein localization characteristics or altered 10 protein half-life compared to the native polypeptide.

As used herein, the term "DRO3 nucleic acid" encompasses nucleic acids with the sequence provided in or complementary to the sequence provided in SEQ ID NO:1, as well as functionally active fragments, derivatives, or orthologs thereof. A DRO3 nucleic acid of this invention may be DNA, derived from genomic DNA or cDNA, or RNA.

15 In one embodiment, a functionally active DRO3 nucleic acid encodes or is complementary to a nucleic acid that encodes a functionally active DRO3 polypeptide. Included within this definition is genomic DNA that serves as a template for a primary RNA transcript (i.e., an mRNA precursor) that requires processing, such as splicing, before encoding the functionally active DRO3 polypeptide. A DRO3 nucleic acid can 20 include other non-coding sequences, which may or may not be transcribed; such sequences include 5' and 3' UTRs, polyadenylation signals and regulatory sequences that control gene expression, among others, as are known in the art. Some polypeptides require processing events, such as proteolytic cleavage, covalent modification, etc., in order to become fully active. Accordingly, functionally active nucleic acids may encode the 25 mature or the pre-processed DRO3 polypeptide, or an intermediate form. A DRO3 polynucleotide can also include heterologous coding sequences, for example, sequences that encode a marker included to facilitate the purification of the fused polypeptide, or a transformation marker.

In another embodiment, a functionally active DRO3 nucleic acid is capable of 30 being used in the generation of loss-of-function DRO3 phenotypes, for instance, via antisense suppression, co-suppression, etc.

In one preferred embodiment, a DRO3 nucleic acid used in the methods of this invention comprises a nucleic acid sequence that encodes or is complementary to a sequence that encodes a DRO3 polypeptide having at least 50%, 60%, 70%, 75%, 80%,

85%, 90%, 95% or more sequence identity to the polypeptide sequence presented in SEQ ID NO:2.

In another embodiment a DRO3 polypeptide of the invention comprises a polypeptide sequence with at least 50% or 60% identity to the DRO3 polypeptide sequence of SEQ ID NO:2, and may have at least 70%, 80%, 85%, 90% or 95% or more sequence identity to the DRO3 polypeptide sequence of SEQ ID NO:2. In another embodiment, a DRO3 polypeptide comprises a polypeptide sequence with at least 50%, 60%, 70%, 80%, 85%, 90% or 95% or more sequence identity to a functionally active fragment of the polypeptide presented in SEQ ID NO:2, such as a TIR domain. In yet another embodiment, a DRO3 polypeptide comprises a polypeptide sequence with at least 50%, 60 %, 70%, 80%, or 90% identity to the polypeptide sequence of SEQ ID NO:2 over its entire length and comprises TIR domain.

In another aspect, a DRO3 polynucleotide sequence is at least 50% to 60% identical over its entire length to the DRO3 nucleic acid sequence presented as SEQ ID NO:1, or nucleic acid sequences that are complementary to such a DRO3 sequence, and may comprise at least 70%, 80%, 85%, 90% or 95% or more sequence identity to the DRO3 sequence presented as SEQ ID NO:1 or a functionally active fragment thereof, or complementary sequences.

As used herein, "percent (%) sequence identity" with respect to a specified subject sequence, or a specified portion thereof, is defined as the percentage of nucleotides or amino acids in the candidate derivative sequence identical with the nucleotides or amino acids in the subject sequence (or specified portion thereof), after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent sequence identity, as generated by the program WU-BLAST-2.0a19 (Altschul *et al.*, J. Mol. Biol. (1990) 215:403-410; website at blast.wustl.edu/blast/README.html) with search parameters set to default values. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched. A "% identity value" is determined by the number of matching identical nucleotides or amino acids divided by the sequence length for which the percent identity is being reported. "Percent (%) amino acid sequence similarity" is determined by doing the same calculation as for determining % amino acid sequence identity, but including conservative amino acid substitutions in addition to identical amino acids in the computation. A conservative amino acid substitution is one in which an amino acid is

substituted for another amino acid having similar properties such that the folding or activity of the protein is not significantly affected. Aromatic amino acids that can be substituted for each other are phenylalanine, tryptophan, and tyrosine; interchangeable hydrophobic amino acids are leucine, isoleucine, methionine, and valine; interchangeable polar amino acids are glutamine and asparagine; interchangeable basic amino acids are arginine, lysine and histidine; interchangeable acidic amino acids are aspartic acid and glutamic acid; and interchangeable small amino acids are alanine, serine, threonine, cysteine and glycine.

Derivative nucleic acid molecules of the subject nucleic acid molecules include sequences that hybridize to the nucleic acid sequence of SEQ ID NO:1. The stringency of hybridization can be controlled by temperature, ionic strength, pH, and the presence of denaturing agents such as formamide during hybridization and washing. Conditions routinely used are well known (see, e.g., Current Protocol in Molecular Biology, Vol. 1, Chap. 2.10, John Wiley & Sons, Publishers (1994); Sambrook *et al., supra*). In some embodiments, a nucleic acid molecule of the invention is capable of hybridizing to a nucleic acid molecule containing the nucleotide sequence of SEQ ID NO:1 under stringent hybridization conditions that comprise: prehybridization of filters containing nucleic acid for 8 hours to overnight at 65° C in a solution comprising 6X single strength citrate (SSC) (1X SSC is 0.15 M NaCl, 0.015 M Na citrate; pH 7.0), 5X Denhardt's solution, 0.05% sodium pyrophosphate and 100 µg/ml herring sperm DNA; hybridization for 18-20 hours at 65° C in a solution containing 6X SSC, 1X Denhardt's solution, 100 µg/ml yeast tRNA and 0.05% sodium pyrophosphate; and washing of filters at 65° C for 1 h in a solution containing 0.2X SSC and 0.1% SDS (sodium dodecyl sulfate). In other embodiments, moderately stringent hybridization conditions are used that comprise: pretreatment of filters containing nucleic acid for 6 h at 40° C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA; hybridization for 18-20 h at 40° C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml salmon sperm DNA, and 10% (wt/vol) dextran sulfate; followed by washing twice for 1 hour at 55° C in a solution containing 2X SSC and 0.1% SDS. Alternatively, low stringency conditions can be used that comprise: incubation for 8 hours to overnight at 37° C in a solution comprising 20% formamide, 5 x SSC, 50 mM sodium phosphate (pH 7.6), 5X Denhardt's solution, 10% dextran sulfate,

and 20 µg/ml denatured sheared salmon sperm DNA; hybridization in the same buffer for 18 to 20 hours; and washing of filters in 1 x SSC at about 37° C for 1 hour.

As a result of the degeneracy of the genetic code, a number of polynucleotide sequences encoding a DRO3 polypeptide can be produced. For example, codons may be selected to increase the rate at which expression of the polypeptide occurs in a particular host species, in accordance with the optimum codon usage dictated by the particular host organism (see, e.g., Nakamura Y et al, Nucleic Acids Res (1999) 27:292). Such sequence variants may be used in the methods of this invention.

- The methods of the invention may use orthologs of the *Arabidopsis* DRO3.
- 10 Methods of identifying the orthologs in other plant species are known in the art. Normally, orthologs in different species retain the same function, due to presence of one or more protein motifs and/or 3-dimensional structures. In evolution, when a gene duplication event follows speciation, a single gene in one species, such as *Arabidopsis*, may correspond to multiple genes (paralogs) in another. As used herein, the term
- 15 "orthologs" encompasses paralogs. When sequence data is available for a particular plant species, orthologs are generally identified by sequence homology analysis, such as BLAST analysis, usually using protein bait sequences. Sequences are assigned as a potential ortholog if the best hit sequence from the forward BLAST result retrieves the original query sequence in the reverse BLAST (Huynen MA and Bork P, Proc Natl Acad Sci (1998) 95:5849-5856; Huynen MA et al., Genome Research (2000) 10:1204-1210).
- 20 Programs for multiple sequence alignment, such as CLUSTAL (Thompson JD et al, Nucleic Acids Res (1994) 22:4673-4680) may be used to highlight conserved regions and/or residues of orthologous proteins and to generate phylogenetic trees. In a phylogenetic tree representing multiple homologous sequences from diverse species (e.g.,
- 25 retrieved through BLAST analysis), orthologous sequences from two species generally appear closest on the tree with respect to all other sequences from these two species. Structural threading or other analysis of protein folding (e.g., using software by ProCeryon, Biosciences, Salzburg, Austria) may also identify potential orthologs. Nucleic acid hybridization methods may also be used to find orthologous genes and are preferred
- 30 when sequence data are not available. Degenerate PCR and screening of cDNA or genomic DNA libraries are common methods for finding related gene sequences and are well known in the art (see, e.g., Sambrook, *supra*; Dieffenbach C and Dveksler G (Eds.) PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY, 1989). For instance, methods for generating a cDNA library from the plant species of interest and

probing the library with partially homologous gene probes are described in Sambrook *et al.* A highly conserved portion of the *Arabidopsis* DRO3 coding sequence may be used as a probe. DRO3 ortholog nucleic acids may hybridize to the nucleic acid of SEQ ID NO:1 under high, moderate, or low stringency conditions. After amplification or isolation of a segment of a putative ortholog, that segment may be cloned and sequenced by standard techniques and utilized as a probe to isolate a complete cDNA or genomic clone.

Alternatively, it is possible to initiate an EST project to generate a database of sequence information for the plant species of interest. In another approach, antibodies that specifically bind known DRO3 polypeptides are used for ortholog isolation. Western blot analysis can determine that a DRO3 ortholog (i.e., an orthologous protein) is present in a crude extract of a particular plant species. When reactivity is observed, the sequence encoding the candidate ortholog may be isolated by screening expression libraries representing the particular plant species. Expression libraries can be constructed in a variety of commercially available vectors, including lambda gt11, as described in Sambrook, *et al.*, *supra*. Once the candidate ortholog(s) are identified by any of these means, candidate orthologous sequence are used as bait (the "query") for the reverse BLAST against sequences from *Arabidopsis* or other species in which DRO3 nucleic acid and/or polypeptide sequences have been identified.

DRO3 nucleic acids and polypeptides may be obtained using any available method. For instance, techniques for isolating cDNA or genomic DNA sequences of interest by screening DNA libraries or by using polymerase chain reaction (PCR), as previously described, are well known in the art. Alternatively, nucleic acid sequence may be synthesized. Any known method, such as site directed mutagenesis (Kunkel TA *et al.*, *Methods Enzymol.* (1991) 204:125-39), may be used to introduce desired changes into a cloned nucleic acid.

In general, the methods of the invention involve incorporating the desired form of the DRO3 nucleic acid into a plant expression vector for transformation of in plant cells, and the DRO3 polypeptide is expressed in the host plant.

An isolated DRO3 nucleic acid molecule is other than in the form or setting in which it is found in nature and is identified and separated from least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the DRO3 nucleic acid. However, an isolated DRO3 nucleic acid molecule includes DRO3 nucleic acid molecules contained in cells that ordinarily express DRO3 where, for

example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

Generation of Genetically Modified Plants with a Drought Tolerance Phenotype

5 DRO3 nucleic acids and polypeptides may be used in the generation of genetically modified plants having a modified, preferably an improved drought tolerance phenotype. Such plants may further display increased tolerance to other abiotic stresses, particular salt-stress and freezing, as responses to these stresses and drought stress are mediated by ABA (Thomashow, 1999 *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50: 571; Cushman
10 and Bohnert, 2000, *Curr. Opin. Plant Biol.* 3: 117; Kang et al. 2002, *Plant Cell* 14:343-357; Quesada et al. 2000, *Genetics* 154: 421; Kasuga et al. 1999, *Nature Biotech.* 17: 287-291).

The methods described herein are generally applicable to all plants. Drought tolerance is an important trait in almost any agricultural crop; most major agricultural crops, including corn, wheat, soybeans, cotton, alfalfa, sugar beets, onions, tomatoes, and beans, are susceptible to drought stress. Although activation tagging and gene identification are carried out in *Arabidopsis*, the DRO3 gene (or an ortholog, variant or fragment thereof) may be expressed in any type of plant. The invention may directed to fruit- and vegetable-bearing plants, plants used in the cut flower industry, grain-producing plants, oil-producing plants, nut-producing plants, crops including corn (*Zea mays*), soybean (*Glycine max*), cotton (*Gossypium*), tomato (*Lycopersicum esculentum*), alfalfa (*Medicago sativa*), flax (*Linum usitatissimum*), tobacco (*Nicotiana*), and turfgrass (*Poaceae* family), and other forage crops, among others.

The skilled artisan will recognize that a wide variety of transformation techniques exist in the art, and new techniques are continually becoming available. Any technique that is suitable for the target host plant can be employed within the scope of the present invention. For example, the constructs can be introduced in a variety of forms including, but not limited to as a strand of DNA, in a plasmid, or in an artificial chromosome. The introduction of the constructs into the target plant cells can be accomplished by a variety of techniques, including, but not limited to *Agrobacterium*-mediated transformation, electroporation, microinjection, microprojectile bombardment calcium-phosphate-DNA co-precipitation or liposome-mediated transformation of a heterologous nucleic acid. The transformation of the plant is preferably permanent, *i.e.* by integration of the introduced expression constructs into the host plant genome, so that the introduced constructs are

passed onto successive plant generations. Depending upon the intended use, a heterologous nucleic acid construct comprising an DRO3 polynucleotide may encode the entire protein or a biologically active portion thereof.

In one embodiment, binary Ti-based vector systems may be used to transfer 5 polynucleotides. Standard *Agrobacterium* binary vectors are known to those of skill in the art, and many are commercially available (e.g., pBI121 Clontech Laboratories, Palo Alto, CA).

The optimal procedure for transformation of plants with *Agrobacterium* vectors will vary with the type of plant being transformed. Exemplary methods for 10 *Agrobacterium*-mediated transformation include transformation of explants of hypocotyl, shoot tip, stem or leaf tissue, derived from sterile seedlings and/or plantlets. Such transformed plants may be reproduced sexually, or by cell or tissue culture. *Agrobacterium* transformation has been previously described for a large number of different types of plants and methods for such transformation may be found in the scientific 15 literature.

Expression (including transcription and translation) of DRO3 may be regulated with respect to the level of expression, the tissue type(s) where expression takes place and/or developmental stage of expression. A number of heterologous regulatory sequences (e.g., promoters and enhancers) are available for controlling the expression of a 20 DRO3 nucleic acid. These include constitutive, inducible and regulatable promoters, as well as promoters and enhancers that control expression in a tissue- or temporal-specific manner. Exemplary constitutive promoters include the raspberry E4 promoter (U.S. Patent Nos. 5,783,393 and 5,783,394), the 35S CaMV (Jones JD *et al.*, (1992) Transgenic Res 1:285-297), the CsVMV promoter (Verdaguer B *et al.*, Plant Mol Biol (1998) 37:1055-1067) and the melon actin promoter (published PCT application WO0056863). Exemplary tissue-specific promoters include the tomato E4 and E8 promoters (U.S. Patent 25 No. 5,859,330) and the tomato 2AII gene promoter (Van Haaren MJJ *et al.*, Plant Mol Bio (1993) 21:625-640).

In one preferred embodiment, DRO3 expression is under control of regulatory 30 sequences from genes whose expression is associated with drought stress. For example, when the promoter of the drought stress responsive *Arabidopsis rd29A* gene was used to drive expression of *DREB1A*, *Arabidopsis* plants were more tolerant to drought, salt and freezing stress and did not have the stunted stature associated with plants over-expressing the *DREB1A* gene from the CaMV 35S promoter (Kasuga *et al.*, 1999 Nature Biotech 17:

287). Promoters from other *Arabidopsis* genes that are responsive to drought stress, such as *COR47* (Welin et al. 1995, *Plant Mol. Biol.* 29: 391), *KIN1* (Kurkela and Franck, 1990, *Plant Mol. Biol.* 15: 137), *RD22BP* (Abe et al. 1997, *Plant Cell* 9, 1859), *ABA1* (Accession Number AAG17703), and *ABA3* (Xiong et al. 2001, *Plant Cell* 13: 2063), could be used.

- 5 Promoters from drought stress inducible genes in other species could be used also. Examples are the *rab17*, *ZmFer1* and *ZmFer2* genes from maize (Bush et al, 1997 *Plant J* 11:1285; Fobis-Loisy, 1995 *Eur J Biochem* 231:609), the *tdi-65* gene from tomato (Harrak, 2001 *Genome* 44:368), the *His1* gene of tobacco (Wei and O'Connell, 1996 *Plant Mol Biol* 30:255), the *Vupat1* gene from cowpea (Matos, 2001 *FEBS Lett* 491:188), and
10 *CDSP34* from *Solanum tuberosum* (Gillet et al, 1998 *Plant J* 16:257).

In yet another aspect, in some cases it may be desirable to inhibit the expression of endogenous DRO3 in a host cell. Exemplary methods for practicing this aspect of the invention include, but are not limited to antisense suppression (Smith, et al., *Nature* (1988) 334:724-726; van der Krol et al., *Biotechniques* (1988) 6:958-976); co-suppression (Napoli, et al, *Plant Cell* (1990) 2:279-289); ribozymes (PCT Publication WO 97/10328); and combinations of sense and antisense (Waterhouse, et al., *Proc. Natl. Acad. Sci. USA* (1998) 95:13959-13964). Methods for the suppression of endogenous sequences in a host cell typically employ the transcription or transcription and translation of at least a portion of the sequence to be suppressed. Such sequences may be homologous to coding as well
15 as non-coding regions of the endogenous sequence. Antisense inhibition may use the entire cDNA sequence (Sheehy et al., *Proc. Natl. Acad. Sci. USA* (1988) 85:8805-8809), a partial cDNA sequence including fragments of 5' coding sequence, (Cannon et al., *Plant Molec. Biol.* (1990) 15:39-47), or 3' non-coding sequences (Ch'ng et al., *Proc. Natl. Acad. Sci. USA* (1989) 86:10006-10010). Cosuppression techniques may use the entire cDNA
20 sequence (Napoli et al., *supra*; van der Krol et al., *The Plant Cell* (1990) 2:291-299), or a partial cDNA sequence (Smith et al., *Mol. Gen. Genetics* (1990) 224:477-481).
25

Standard molecular and genetic tests may be performed to further analyze the association between a gene and an observed phenotype. Exemplary techniques are
30 described below.

1. DNA/RNA analysis

The stage- and tissue-specific gene expression patterns in mutant versus wild-type lines may be determined, for instance, by *in situ* hybridization. Analysis of the methylation status of the gene, especially flanking regulatory regions, may be performed. Other

suitable techniques include overexpression, ectopic expression, expression in other plant species and gene knock-out (reverse genetics, targeted knock-out, viral induced gene silencing [VIGS, see Baulcombe D, *Arch Virol Suppl* (1999) 15:189-201]).

In a preferred application expression profiling, generally by microarray analysis, is used to simultaneously measure differences or induced changes in the expression of many different genes. Techniques for microarray analysis are well known in the art (Schena M *et al.*, *Science* (1995) 270:467-470; Baldwin D *et al.*, (1999) *Cur Opin Plant Biol.* 2(2):96-103; Dangond F, *Physiol Genomics* (2000) 2:53-58; van Hal NL *et al.*, *J Biotechnol* (2000) 78:271-280; Richmond T and Somerville S, *Curr Opin Plant Biol* (2000) 3:108-116). Expression profiling of individual tagged lines may be performed. Such analysis can identify other genes that are coordinately regulated as a consequence of the overexpression of the gene of interest, which may help to place an unknown gene in a particular pathway.

2. Gene Product Analysis

Analysis of gene products may include recombinant protein expression, antisera production, immunolocalization, biochemical assays for catalytic or other activity, analysis of phosphorylation status, and analysis of interaction with other proteins via yeast two-hybrid assays.

3. Pathway Analysis

Pathway analysis may include placing a gene or gene product within a particular biochemical, metabolic or signaling pathway based on its mis-expression phenotype or by sequence homology with related genes. Alternatively, analysis may comprise genetic crosses with wild-type lines and other mutant lines (creating double mutants) to order the gene in a pathway, or determining the effect of a mutation on expression of downstream "reporter" genes in a pathway.

Generation of Mutated Plants with a Drought Tolerant Phenotype

The invention further provides a method of identifying plants that have mutations in endogenous DRO3 that confer increased drought tolerance, and generating drought-tolerant progeny of these plants that are not genetically modified. In one method, called "TILLING" (for targeting induced local lesions in genomes), mutations are induced in the seed of a plant of interest, for example, using EMS treatment. The resulting plants are grown and self-fertilized, and the progeny are used to prepare DNA samples. DRO3-specific PCR is used to identify whether a mutated plant has a DRO3 mutation. Plants

having DRO3 mutations may then be tested for drought tolerance, or alternatively, plants may be tested for drought tolerance, and then DRO3-specific PCR is used to determine whether a plant having increased drought tolerance has a mutated DRO3 gene. TILLING can identify mutations that may alter the expression of specific genes or the activity of proteins encoded by these genes (see Colbert et al (2001) Plant Physiol 126:480-484; McCallum et al (2000) Nature Biotechnology 18:455-457).

In another method, a candidate gene/Quantitative Trait Locus (QTLs) approach can be used in a marker-assisted breeding program to identify alleles of or mutations in the DRO3 gene or orthologs of DRO3 that may confer increased tolerance to drought (see 10 Foolad et al., Theor Appl Genet. (2002) 104(6-7):945-958; Rothan et al., Theor Appl Genet (2002) 105(1):145-159); Dekkers and Hospital, Nat Rev Genet. (2002) Jan;3(1):22-32). Thus, in a further aspect of the invention, a DRO3 nucleic acid is used to identify whether a drought-tolerant plant has a mutation in endogenous DRO3.

While the invention has been described with reference to specific methods and 15 embodiments, it will be appreciated that various modifications and changes may be made without departing from the invention. All publications cited herein are expressly incorporated herein by reference for the purpose of describing and disclosing compositions and methodologies that might be used in connection with the invention. All cited patents, patent applications, and sequence information in referenced websites and public databases 20 are also incorporated by reference.

EXAMPLES

EXAMPLE 1

Generation of Plants with a DRO3 Phenotype by Transformation with an Activation 25 Tagging Construct

Mutants were generated using the activation tagging "ACTTAG" vector, pSKI015 (GI 6537289; Weigel D et al., *supra*). Standard methods were used for the generation of *Arabidopsis* transgenic plants, and were essentially as described in published application PCT WO183697. Briefly, T0 *Arabidopsis* (Col-0) plants were transformed with 30 *Agrobacterium* carrying the pSKI015 vector, which comprises T-DNA derived from the *Agrobacterium* Ti plasmid, an herbicide resistance selectable marker gene, and the 4X CaMV 35S enhancer element. Transgenic plants were selected at the T1 generation based on herbicide resistance. T2 seed was collected from T1 plants and stored in an indexed collection, and a portion of the T2 seed was accessed for the screen.

Approximately 18 T2 seed from each of line tested were planted in soil. The seed were stratified for 3 days at 4°C and then grown in the greenhouse. Plants containing the ACTTAG insert were selected. Drought stress was imposed on the selected plants by withholding water for 21-25 days. At this time the plants were beginning to bolt.

5 From 6 to 10 days after the initiation of drought stress, observations of the plants were taken daily. Transgenic plants were compared to each other and wild-type control plants. Putative drought tolerant lines were identified as containing at least 2 plants that remained green and viable after wild-type plants had died or as plants that contained a soil moisture content of at least 50 mV (using a Delt-T Devices HH2 Soil Moisture Meter with
10 a ML2x Theta Probe).

After drought tolerant lines were identified, water was applied to the plants to allow them to recover. When possible, T3 seed was collected from the plants. This T3 seed was then grown and the plants assessed for drought tolerant phenotype as described above.

15 The drought tolerant plants were then subjected to an "excised leaf transpiration test" in which seeds were planted, stratified, and grown for three weeks as described above. Then, either the entire rosette or a single leaf was excised and placed on a pre-weighed plastic weigh dish and left on the bench at room temperature. The mass of the plant material was recorded immediately after excision and at 30 min intervals afterward.
20 The mass of drought tolerant plants often decreased less rapidly indicating that they were transpiring less rapidly.

To detect lines containing or lacking the insert, PCR analysis was performed using a set of DNA oligonucleotide primers; one that hybridizes to sequences in pSKI015, the other that hybridizes to sequences flanking the insert. Genotyping of individuals analyzed
25 drought tolerance experiments indicated that plants containing the insert identified in W000095647 were more tolerant of drought stress than plants without the insert.

EXAMPLE 2

Characterization of the T-DNA Insertion in Plants Exhibiting the Altered Drought Tolerance Phenotype.

We performed standard molecular analyses, essentially as described in patent application PCT WO0183697, to determine the site of the T-DNA insertion associated with the increased drought tolerance phenotype. Briefly, genomic DNA was extracted from plants exhibiting increased drought tolerance. PCR, using primers specific to the

pSKI015 vector, confirmed the presence of the 35S enhancer in plants from line W000095647, and Southern blot analysis verified the genomic integration of the ACTTAG T-DNA and showed the presence of a single T-DNA insertion in the transgenic line.

5 Plasmid rescue was used to recover genomic DNA flanking the T-DNA insertion, which was then subjected to sequence analysis.

The sequence flanking the left T-DNA border was subjected to a basic BLASTN search and/or a search of the *Arabidopsis* Information Resource (TAIR) database (available at the arabidopsis.org website), which revealed sequence identity to nucleotides 10 71190-71750 on Arabidopsis BAC clone F14G24 (GI 12324627), mapped to chromosome 1. Sequence analysis revealed that the T-DNA had inserted 1501 base pairs from the start codon in At1g52900, whose nucleotide sequence is presented as SEQ ID NO: 1 and GI 18404238, and which we designated DRO3.

15 **EXAMPLE 3**

Analysis of *Arabidopsis* DRO3 Sequence

The amino acid sequence predicted from the DRO3 nucleic acid sequence is presented in SEQ ID NO:2 and GI 15219124.

Sequence analyses were performed with BLAST (Altschul *et al.*, 1990, J. Mol. Biol. 215:403-410) and PFAM (Bateman *et al.*, *supra*), PSORT (Nakai K, and Horton P, 1999, Trends Biochem Sci 24:34-6), among others. PFAM analysis predicted a TIR domain (PF01582) from amino acids 40 to 149. The TIR domain is an intracellular signaling domain found in MyD88, interleukin 1 receptor, and the Toll receptor.

Orthologs of the *Arabidopsis* DRO3 that can be used in the practice of the invention are identified in Table 1 below.

TABLE 1

Ortholog Gene Name	Species	GI #	% ID to <i>Arabidopsis</i> DRO3	Coordinates of protein motif (s)
OJ1773_H_01.25	Oryza sativa	gi 24417199	Identities = 79/161 (49%), Positives = 118/161 (73%)	PF01582: amino acids 61 to 182

	Solanum tuberosum subsp. andigena	gi 16944811	Identities = 63/175 (36%), Positives = 95/175 (54%)	PF01582: amino acids 25 to 161.
	Pinus taeda	gi 21655205	Identities = 57/162 (35%), Positives = 89/162 (54%)	PF01582: amino acids 26 to 165.
	Pinus taeda	gi 21655199	Identities = 54/166 (32%), Positives = 89/166 (53%)	PF01582: amino acids 14 to 143.
GS0-2	Glycine max	gi 22037313	Identities = 46/144 (31%), Positives = 79/144 (54%)	PF01582: amino acids 14 to 146.
NBS2	Helianthus annuus	gi 15787891	Identities = 62/204 (30%), Positives = 102/204 (50%)	PF01582: amino acids 39 to 169.
R 13 protein	Glycine max	gi 27764543	Identities = 55/164 (33%), Positives = 87/164 (53%)	PF01582: amino acids 14 to 146.
	Populus balsamifera subsp. trichocarpa	gi 23477203	Identities = 49/170 (28%), Positives = 92/170 (54%)	PF01582: amino acids 17 to 149.

Additionally, a translation of an EST contig from *Glycine max*, presented as SEQ ID NO:3, shares 60% sequence identity with SEQ ID NO:2.

5

EXAMPLE 4

Confirmation of Phenotype/Genotype Association

- RT-PCR analysis showed that the DRO3 gene was specifically overexpressed in plants from the line displaying the improved drought tolerance phenotype. Specifically, RNA was extracted from tissues derived from plants exhibiting the DRO3 phenotype and from wild type COL-0 plants. RT-PCR was performed using primers specific to the sequence presented as SEQ ID NO:1 and to other predicted genes in the vicinity of the T-DNA insertion. The results showed that plants displaying the DRO3 phenotype over-expressed the mRNA for the DRO3 gene by about 1,000 fold compared to wild type plants, indicating that the enhanced expression of the DRO3 gene is correlated with the DRO3 phenotype.